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IN VITRO ANTIFUNGAL ACTIVITY OF METHANOLIC EXTRACTS OF DIFFERENT *Senna didymobotrya* (FRESEN.) H.S. IRWIN & BARNEBY PLANT PARTS

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Abstract

Background: Herbal medicines have been in use for many years and remain widespread in developing countries; whereas, the use of complementary alternative medicine is on the increase in developed countries. *Senna didymobotrya* is important for its medicinal benefits among most communities in treating a wide range of ailments.
Materials and methods: Plants were collected from a cluster in Siaya, Nandi and Nakuru counties (Kenya). Stem bark, root bark, leaves, flowers and immature pods were obtained; air-dried and ground into fine powder. Methanol was used to extract the plant extracts. The extracts were reconstituted in water and incorporated into growth media to obtain 0%, 2.5%, 5%, 7.5% and 10%. Bioassays were carried out on *T. tonsurans* (ATCC 28942) and *C. albicans* (14053). The growth of cultures on the plates was measured over a period of sixteen days. The area under disease progress stairs was determined and subjected to ANOVA and comparison of means using LSD.
Results: Results indicated that the growth of *C. albicans* was not significantly affected by the plant extracts. Growth of *T. tonsurans* was completely inhibited by immature pods extract at 10%, the leaves and flowers extracts inhibited the growth at 7.5%. The stem and root bark extracts inhibited growth at low dosages of 2.5- 5 %.
Conclusion: There is need to carry out research on root and stem barks to identify the active phytochemicals that contribute to their high efficacies. On species conservation, harvesting of roots may lead to depletion of *S. didymobotrya*.

Key words: *Senna didymobotrya*, ringworms, candidiasis, tinea capitis

Introduction

Herbal medicines have been in use over the years in many parts of the world especially within developing countries (Kareru et al., 2007a; Gabriel et al., 2016). Despite remarkable advancements in conventional medicines, traditional medicines have been recognised by the World Health Organization (WHO, 2002) as a building block of primary healthcare (Patil and Saini, 2012), partly because some conventional drugs have failed to prove effective or have serious side effects (Njoroge and Bussmann, 2006; Bhadauria and Kumar, 2011). Significant body of evidence has continue to demonstrate the promising potential of medicinal plants used in various traditional, complementary and alternative systems of treatment of human diseases (id and Akhtar,2016; Shabir et al., 2015;Wong and Cuervo, 2010;). The world bank has recently made a strong case for herbal healthcare (Kareru et al., 2007b), having recognized the vital values of medicinal plants. These values include: medicinal, ecological, income generation, cultural, social and religious roles. In tropical countries, modern medicines are not affordable to most rural dwellers and WHO estimates that 80% of the world's population use botanical medicines for primary health care (Muregi et al., 2004). The World Bank report further pointed out that in 2002, Kenya's Ministry of Health budget for medicine provided for only 30% of the population (WHO, 2002). This left 70% (21 million) of the population which could not access the conventional medicines. The latter population group was therefore left to rely on traditional medicines for their healthcare needs (Sharma and Alagarsamy, 2012).

Senna didymobotrya, African *senna*, peanut butter cassia (Cherono and Akoo, 2011) is a shrub widely distributed in East Africa and widely used as medicinal plant in treating fungal skin infections in Kenya. Humphries and Hughes (2006) reported that extracts of both *S. didymobotrya* and *S. occidentalis* have antifungal activity by inhibition of mycelial growth and aflatoxin formation. It is widely used as a medicinal plant, especially in East Africa, where a decoction or infusion from the leaves, stems and roots is drunk as a laxative and purgative for the treatment of abdominal pains. In Uganda, Rwanda and Burundi it is also taken to expel intestinal worms and to treat ringworms. In DR Congo, Rwanda, Burundi, Kenya, Uganda and Tanzania a root decoction of *S. didymobotrya* is drunk for the treatment of malaria, other fevers and jaundice (Nyaberi et al., 2013). A survey on medicinal plants used in the treatment of fungal and bacterial infections in western Uganda documented sixty seven plants and *S. didymobotrya* leaves and roots were utilized (Kamatenesi - Mugisha et al., 2008). Tabuti (2008) indicated its use in treatment of malaria.Pounded leaves and young stems are used in Kenya to treat skin diseases. The pulp is applied to the skin (Gachathi, 1989). *S. didymobotrya* is widely used by traditional healers among the Embu and Mbeere to treat ring-worm and other skin diseases (Adongo et al., 2012). In their studies in Central Province of Kenya, they reported that ringworm and candidiasis were the most common fungal infections. In the same survey *S. didymobotrya* was one of the most highly utilized plant species for management of various skin conditions. In Kipsigis community, it's used in the management of opportunistic fungal infections, malaria, diarrhoea, skin infections in humans (Bii et al., 2003 and Korir et al., 2012). The objective of this study was to assess, *in vitro*, the efficacy of different concentrations of *S. didymobotrya* plant extracts on the fungal species *Candida albicans* and *Trichophyton tonsurans* disease causative agents of candidiasis and tinea capitis respectively.

Materials and Methods

Plant samples were collected from three geographic regions (locations) in Kenya. These were Uyawi-Miandhe in Siaya county, Ndurio in Nandi county and Rongai in Nakuru county. These areas represent the different ecological zones that *Senna* was found to be growing in the wild. The samples were collected following standard and ethical procedures (WHO, 2003). At each location, 20 mature plants from a cluster were selected. From these plants samples of the young leaves, flowers, stem bark, immature pods and root barks were obtained and separately packed in gunny bags. Samples were dried under a shade at 25 – 30^{o} C for two weeks. Dried samples were shredded using an electric hammer mill before grinding using the laboratory Willy mill at 800 rpm.

Extraction

The methanolic extraction was carried out according to methods described by Kigondu et al. (2009) with modifications. Using an electric analytical beam balance, 1000 grams of powdered dried plant part was weighed. At each time, 42.25 g was placed in soxhlet apparatus wrapped in a filter paper and distilled with absolute methanol. The extracted subsamples were mixed in a conical flask then placed in a rotary vacuum evaporator in a water bath at 40^{o}C to recover the solvent (methanol) and concentrate the crude extract. The semi-solid extract was placed in sterile beaker and left in the laminar flow hood for 24 hours for complete evaporation of the solvent. The total yield of the solid crude extract was weighed and put in a tightly screwed capped glass containers and stored in the refrigerator at 4^{o}C prior to use for biological assays.

Potato dextrose agar (PDA) and sabouraud dextrose agar (SDA) were prepared according to manufacturers' instructions. After autoclaving at121^{o}C for 15 minutes each medium was allowed to cool to 50^{o}C. Using a sterile measuring cylinder, 100 ml of medium was measured and poured into 100 ml Erlenmeyer flasks placed in the laminar flow hood.

The constitutions of the extracts were carried out according to methods of Akanwariwiak et al. (2012) and Chad (2013) with modifications. Using analytical balance, 2.5 g, 5 g, 7.5 g and 10 g of crude extract was weighed and placed in sterile beakers. Five millilitres of warm sterile water was then added to the solid extract to reconstitute it. Each of the reconstituted extract was added into 100 ml of respective medium in a conical flask, stirred and let to stand at 40-50^{o}C for 30 min. The positive control consisted of ketoconazole (Keto) incorporated at 10µg/ml of media (Heeres et al., 1979).The medium in each flask was poured in four petri dishes (replicates).

The test microorganisms: (a) *Candida albicans* (ATCC 1405) and (b) *Trichophyton tonsurans* (ATCC 28942) were obtained from Kenya Medical Research Institute (KEMRI), Kisumu, Kenya.*Candida albicans* was cultured in potato dextrose agar (PDA) while *T. tonsurans* was grown in sabouraud dextrose agar (SDA). Fungal cultures were obtained from the plates and a spore suspension of the respective isolate was prepared. Inoculum was placed in 50ml sterile distilled water in a test tube. From this, serial dilution was made to 10^{-3}. Using a haemocytometer slide the spore concentration in the last dilution was determined. The concentration was adjusted to10^{8} spores/ml. From each spore suspension of the two fungal species, 50 µl of the inoculum was inoculated as a drop on the respective agar medium. The experiment had four replicates in a completely randomized design. The diameters of the growing colonies were measured at an interval of four days. Four observations were made.

Data analysis

The diameter (mm) of the colony size were used to calculate the actual surface area (mm^2) covered by the pathogen. The disease areas for the different observations were summarized using the area under disease progress stairs (AUDPS) (Simko and Piepho, 2012):

$$AUDPS = AUDPC + \left[\frac{y_1 + y_n}{2} \times \frac{D}{n-1}\right]$$

Where AUDPC is area under disease progress curve given with formula;
AUDPC = [½ Obs_1t_1 + Obs_2t_2+Obs_3t_3+½Obs_4t_4]
Where:
y_1 and y_n are assessments at the first and last observations respectively.
D = t_n- t_1 (when observations are performed at every time unit, then D = (n -1)
t_1, t_2, t_3 and t_4 are the time intervals of the first, second, third and the fourth observation.
Obs_1, Obs_2, Obs_3 and Obs_4 are first, second, third and fourth observation respectively.
*n*is the total number of observation.
The AUDPS was subjected to Analysis of Variance (ANOVA) and comparison of means was carried out on all data using SAS (SAS statistical package Release 8.02 copy 1999 – 2001). Means were separated by the use of the least significant difference and compared at P = 0.05 for significance. The values are represented as means for AUDPS. Paired t - test was used for reporting the p value and significance with respect to the control.

Results

Results presented in Table 1 below indicated that in Nandi County, root bark yielded the highest percentage of crude extract of 34.8%. This was significantly different (P < 0.05) from the yield of the other plant parts. Immature pods yielded 8.1% and were the lowest extraction from the location. In Nakuru County, the leaves crude extract was highest at 33.2%. Immature pods produced 5.5% crude extract. Samples obtained from Siaya county showed that root bark yielded the highest amount of crude extract of 30.4% whilst flowers and immature pods yielded 1.6% and 6.9% respectively.

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Table 1: The percentage (%) yield of crude extracts obtained from different plant parts of *S. didymobotrya* from three locations

Plant part	Location			
	Nandi	Nakuru	Siaya	Mean
Leaves	28.6 c	33.2 a	24.2 b	28.7
Root bark	34.8 a	20.1 b	30.4 a	28.4
Stem bark	31.4 b	15.0 c	24.5 b	23.6
Flowers	12.4 d	14.4 d	1.6 d	9.5
Immature pods	8.1 e	5.5 e	6.9 c	6.8
Mean	23.1	17.6	17.5	

LSD = 1.3
Means followed by the same letter in a column are not significantly different (P > 0.05).

Candida albicans

Results presented in Table 2 indicated that the flower extracts from Siaya had the highest growth of colonies with a mean AUDPS of 3224.0. Those from Nandi had AUDPS of 1862.1 while Nakuru had 1720.1. These values were not significantly different. A similar trend was observed with stem bark samples in that Siaya plant samples had the highest AUDPS (1969.7) and Nakuru the lowest growth of colonies (950.2).The pods obtained Siaya, Nandi and Nakuru had mean colony AUDPS of 3101.8, 3023.4 and 2690.9 respectively. These observations were not significantly different (P > 0.05). The leaves from Siaya had the highest growth of colonies with mean AUDPS of 2615.6 while leaves from Nandi had AUDPS of 2592.1.These values were not significantly different. The leaves from Nakuru had the lowest growth of colonies of 1840.0.A similar trend was observed with root bark samples. Siaya plant samples in had the highest AUDPS (1624.4) followed by Nandi samples (1514.6 and Nakuru with the lowest AUDPS of 931.5 (Table 2).

Table 2: The Area Under Disease Progress Stairs (AUDPS) of growth of *C. albicans* on culture media impregnated with different concentrations of *S. didymobotrya* obtained from different plant parts and locations

Plant part	Location		
	Siaya	Nandi	Nakuru
Flowers	3224.0 a	1862.1 b	1720.1b
Pods	3101.8 a	3023.4 a	2690.9 a
Leaves	2615.6 a	2592.1 a	1840.0 b
Stem barks	1969.7 a	1133.9 b	950.2 b
Root barks	1624.4 a	1514.6 a	931.5 b
Mean	2507.1	2025.2	1626.5

LSD = 445.5
Means followed by the same letter in a row are not significantly different (P>0.05).

Table 3: The Area Under Disease Progress Stairs (AUDPS) of growth of *C. Albicans* on culture media impregnated with different concentrations of *S. didymobotrya* obtained from different plant parts

Plant part	Concentration (%)					
	0	2.5	5.0	7.5	10.0	Ketoconazole (control)
Flowers	3154.3a	2812.5a	2360.6b	1986.3b	1855.7a	0 a
Pods	3361.6a	2922.9a	2833.5a	2697.3a	1878.4 a	0 a
Leaves	2517.7b	2520.2a	2466.8a	2273.7ab	1868.7 a	0 a
Stem barks	2491.6b	1344.8b	1007.1c	1102.8c	801.2b	0 a
Root barks	2584.2b	1738.3b	843.7c	803.7c	114.3c	0 a
Mean	2821.9	2267.7	1902.3	1772.8	1303.6	0

LSD = 445.5
Means followed by the same letter in a column are not significantly different (p>0.05) using LSD

The negative control treatments had a mean range colony area colony growth range of 2491.6-3361.6 with mean colony area of 2821.9. There were no colony growths of *C. albicans* observed on plates inoculated with Ketoconazole at 10µg/ml. The media treated with 2.5, 5, 7.5 and 10%stem bark and root bark had lower colony growth as compared to the negative control (Table 3). The medium impregnated with 2.5% extract of stem bark and root bark had AUDPS of 1344.8 and 1738.3 respectively. These were not significantly different (P≥0.05). Leaves, flowers and immature pods had AUDPS range of 2520.2- 2812.5. At concentration 5%, the AUDPS for stem bark was 1007.1 whilst that of the root bark was 843.7. They were not significantly different (P≥0.05). The same trend was observed at concentration of 10%. Immature pods, leaves and flowers were not effective as from 2.5%, 5%, 7%, and

10% as compared to the control (Table 3) above. A decrease in colony size was observed in all extract treatments with increase in inoculum concentration.

Trichophyton tonsurans

Results presented in Table 4 showed that from the samples collected in Nakuru county; the flower sample had the highest growth of colonies with a mean AUDPS of 4664.4 followed by Nandi sample with a mean 4603.4. The flowers from Siaya had the lowest growth of colonies with a mean AUDPS of 3273.0 which was significantly different from those of other areas. A similar trend was observed with leaf extracts though samples collected from Nandi county had the highest growth of colonies with mean AUDPS of 5426.7 followed by Nakuru samples (5242.3) while Siaya had the least with AUDPS of 4171.4. The pods from Nandi had the highest colony growth of 5639.8 while those from Siaya and Nakuru Counties were not significantly different with AUDPS values of 4964.2 and 4781.8 respectively. The stem bark samples from Nandi County had the highest AUDPS of 4831.4 followed Siaya samples (3467.6) and Nakuru with the lowest growth of colonies (2805.7). The root bark obtained from Siaya and Nandi had mean colony AUDPS of 2947.6 and 3367.9 respectively. These observations were not significantly different (P > 0.05). The root bark from Nakuru had the lowest growth of colonies with mean AUDPS of 2649.2.

Table 4: The Area Under Disease Progress Stairs (AUDPS) of growth of *T. tonsurans* on culture media impregnated with different concentrations of *S. didymobotrya* obtained from different plant parts and locations

	Location					
Plant part	Siaya		Nandi		Nakuru	
Flowers	3273.0	b	4603.4	a	4664.4	a
Pods	4964.2	b	5639.8	a	4781.8	b
Leaves	4171.4	b	5426.7	a	5242.3	a
Stem bark	3467.6	b	4831.4	a	2805.7	c
Root bark	2947.6	a	3367.9	a	2649.2	b
Mean	3764.8		4773.8		4028.7	

LSD = 653.5
Means followed by the same letter in a row are not significantly different (P>0.05) using LSD

The results in Table 5 indicated that the negative control (0%) had a mean range colony area of colony growth of 13124.2-15834. with mean colony area of 14555.0. The media treated with concentrations of 2.5% of root bark extract showed the lowest growth of 778.0 while the stem bark had the second lowest growth of 2673.2. Immature pods, leaves and flower extracts had lower colony growths as compared to the negative control. The same trend was observed at 5%. Media impregnated with 5% of stem and root bark extracts showed no growth of *T. tonsurans*. There was no colony growth on media impregnated with 7.5 and 10% concentration of all the plant parts extracts (flowers, pods, leaves, stem and root barks). No colony growth was observes on media treated with Ketoconazole.

Table 5: The Area under Disease Progress Stairs (AUDPS) of growth of *T. tonsurans* on culture media impregnated with different concentrations of *S. didymobotrya* obtained from different plant parts

	Concentration (%)					
Plant part	0	2.5	5.0	7.5	10.0	Ketoconazole (control)
Flowers	13124.2d	5664.8b	2112.3b	0 a	0 a	0 a
Pods	15020.4 ab	6829.8a	3792.7a	0 a	0 a	0 a
Leaves	14632.4bc	7621.8a	2479.7b	0 a	0 a	0 a
Stem bark	15834.8 a	2673.2c	0c	0 a	0 a	0 a
Root bark	14163.1c	778.0d	0c	0 a	0 a	0 a
Mean	14555.0	4713.5	1677.0	0	0	0

LSD = 653.5
Means followed by the same letter in a column are not significantly different (P>.0.05) using LSD

Discussion

Extraction of bioactive plant crude extracts

Many plant species have been investigated for antimicrobial activity. Other authors have shown that some plant extracts were used in the management of skin diseases within traditional communities possess antioxidant, haemostatic, analgesic properties as well as immune stimulating activities (Inngjerdingen et al., 2004; Houghton et al., 2005). Figueiredo et al. (2008) stated that mixtures of volatiles and essential oils are slightly soluble in water and highly soluble in organic solvents. Nyaberi et al. (2013) used methanol, chloroform and water to extract phytochemicals from stem charcoal of *S. didymobotrya*. They used cold extraction method and found that methanol was a better extracting solvent of non-medium polar plant compounds than chloroform. Water had the least capability therefore, recommending the use of methanol as a solvent rather than water. Nkere and Iroegbu, (2005) in their

studies found also that methanol was a better solvent on *Picralima nitida* stem bark extracts. Korir et al. (2012) also found that methanol extracts of the stem bark of *S. didymobotrya* yielded higher crude extracts as compared to other organic solvents. Traditional healers use cold water extraction methods to extract the active ingredients from medicinal plants for their use. It is most likely that this method of extraction is not as efficient and they may be extracting less of the active ingredients from the plant parts. In this study we used methanolic extraction method which also enabled us to precisely determine the extract concentrations used unlike when water is used for extraction.

C. albicans

C. albicans is a yeast (fungus) that causes candidiasis. This yeast can live as harmless commensal in many different body locations like the skin, in the intestines and in the vagina, and is carried in almost half of the population (Van Wyk et al., 2002). However, in response to a change in the host environment, *C. albicans* can convert from a benign commensal into a disease-causing pathogen, causing infections in the oral, gastrointestinal and genital tracts. Results presented in Table 2 indicated that the efficacy of the extracts obtained from the different regions did not vary very much. Studies carried out by Pascaline Jeruto (unpublished data) indicated that *S. didimobotrya* populations collected from Siaya, Nakuru and Nandi counties did not vary phenotypically. Results presented in Tables 3 and 6 indicated that at 10% extract concentration none of the plant parts extracts reduced the pathogen colony size by 95%. This means that *S. didymobotrya* plant extracts were not effective against *C. albicans* at the concentrations tested. There were indications that with increased concentrations the AUDPS decreased especially with stem and root barks. There is need to test these extracts at higher dosages above 10%. Other medicinal plants have been found to have effective activity against *C. albicans*. The dichloromethane (DCM): methanol (1: 1) extracts of *Phyllanthus amarus* and *Phyllanthus odontadenius* have been reported with strong antimicrobial activity against *C. albicans* (Njoroge et al., 2012).

Decoctions of the leaves of *Dodonaea viscosa* var. *angustifolia* (hop bush) have been used for the treatment of oral infections (Van Wyk et al., 2002; Patel and Coogan, 2008). *Glycyrrhiza glabra* (liquorice) and *Polygala senega (Seneca snakeroot)* are used extensively in Europe as treatment for oral candidiasis as both plants as been reported to contain saponins compounds known to possess antifungal activity (Van Wyk et al., 2009). Flavonoids isolated from *Eysenhardtia texana* and *Termanalia bellerica* have been reported to possess antifungal activity against *Candida albicans* (Cushnie and Lamb, 2005). Possibly *S. didymobotrya* extracts did not have sufficient amount of these biochemical agents to be effective against *C. albicans*. More studies need to be carried out to determine the quantities of the biochemical in the plant parts.

Table 6: The mean Area under Disease Progress Stairs (AUDPS), the 95% and 99% reductions of the mean colony sizes of the four test organisms

Test organism	Mean colony size	95% reduction in mean colony size	99% reduction in mean colony size
Candida albicans	2823.1	141.2	28.2
Trichophytontonsurans	14555.0	727.8	145.6

T. tonsurans

Tinea capitis (also known as "Herpes tonsurans", "Ringworm of the hair," "Ringworm of the scalp," "Scalp ringworm", and "Tinea tonsurans") is a worldwide problem fungal infection (dermatophytosis) of the scalp. The disease is primarily caused by dermatophytes in the *Trichophyton* and *Microsporum* genera that invade the hair shaft. Studies carried out by Ayaya et al (2001) in Western and Rift Valley regions of Kenya showed that the prevalence of tinea capitis in primary schools was prevalent. The most common cause of tinea capitis was *T. tonsurans*. It is primarily a disease in young children where, males are more infected than females.This may be due to shortness of the hair, which facilitates easy reach of the fungal spores to the scalp.

Several pharmaceutical drugs have been used in tinea treatment which is subjected to a confirmatory test by microscopy/culture. Drugs like terbinafine 250mg, nystatin, and use of selenium or ketoconazole shampoo. Oral antifungal agents like terbinafine, fluconazole and itraconazole are used in treatment of tinea capitis. Griseofulvin is known as the gold standard therapy for tinea capitis and its efficacy is decreasing with time (Shemer et al, 2012). Results presented in this study indicated that immature pods extracts reduced the colony size by 100% at the concentration of 10%. Leaves and flower extracts reduced the colony size by 100% at the concentration of 7.5% (Tables 4 and 6). Root bark and stem bark extracts reduced the colony size by 100% at the concentrations of 7.5% and 5% respectively indicating that these plant parts had higher concentrations of the biochemical agents that were effective against *T. tonsurans*. The root extract was the best in suppression of colony growth and the immature pods extract was the least effective. These results show that *S. didymobotrya* can be used in management of diseases cause by *T. tonsurans* and related species. Pharmacological studies by various groups of investigators have shown that *S. didymobotrya* possesses significant biological activity, such as antibacterial, antibiofilm, antifungal and antioxidant properties. The antimicrobial activity of plant extracts can be attributed to not only a single bioactive principle but also due to the combined action of other compounds (Sunayana et al, 2003). Other authors have shown that some plant extracts used in management of skin diseases in traditional communities' possess antioxidant, haemostatic, analgesic properties as well as immune stimulating activities (Inngjerdingen et al., 2004; Houghton et al., 2005).

S. didymobotrya stem bark and root barks have shown effective antimicrobial activities against *T. tonsurans* at low dosages. There is need to carry our research on these plant part extracts to identify the active phytochemicals that contribute to their high efficacies as compared to leaf, flower and immature pods extracts. On the conservation front, harvesting of these plant parts may lead to depletion of *S. didymobotrya* because they are potentially destructive unlike harvesting of the leaves, flowers and immature pods that regenerate every season. Research should focus on the concentration of the active ingredients in the leaves, flowers and immature pods so as to increase their efficacy. It was observed that *S. didymobotrya* plant extracts were not relatively effective against *Candida albicans*.

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